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Immunology

Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells

(Vβ element/enterotoxins/human T-cell receptors/quantitative polymerase chain reaction)

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ABSTRACT **A modification of the polymerase chain reaction has been used to establish the fact that a collection of *Staphylococcus aureus* toxins are "superantigens," each of which interacts with the T-cell αβ receptor of human T cells by means of a specific set of Vβ elements.**

The antigen receptor [T-cell receptor (TCR)] on most peripheral T cells is a heterodimer made up of α and β chains. Five germ-line-encoded variable elements (Vα, Jα, Vβ, Dβ, and Jβ) as well as non-germ-line-encoded amino acids contribute to the receptor combining site (1–4). The ligands for αβ TCRs are combinations of antigen-derived peptides, bound to major histocompatibility complex proteins (MHC) (5–8). Usually the specificity of TCRs for antigen plus MHC is determined by all of the variable elements of both α and β chains (1–4). Exceptions to this rule have, however, recently been documented by us and others (9–18). In the examples studied so far, the exceptions involve an antigen/MHC combination that can stimulate T cells bearing a particular Vβ, almost regardless of the composition of the rest of the receptor on these cells. We have suggested the term "superantigen" to describe receptor ligands of this type. These superantigens and the Vβ elements that engage them have been well documented in mice with the help of an increasing number of Vβ-specific antibodies and DNA probes.

Some superantigens are endogenously synthesized—for example, a mouse B-cell-derived self-superantigen bound to IE reacts with nearly all T cells bearing Vβ17a and mice that express IE delete nearly all Vβ17a$^+$ T cells (9, 10). Exogenous superantigens have also been described. We and others have recently shown that some bacterial proteins are also able to stimulate T cells in a Vβ-specific fashion (15–19). For example, *Staphylococcus aureus* enterotoxin B (SEB) stimulates mouse T cells bearing Vβ3, -7, -8.1, -8.2, -8.3, and -17 and has no effect on most other cells. Since the *S. aureus* toxins are important contributors to morbidity and mortality in man (20), we wished to extend our studies on bacterial superantigens to human T cells. In an initial study, we used monoclonal antibodies (mAbs) to four human Vβ elements to show that T cells expressing these Vβ elements were differentially stimulated by a panel of *S. aureus* toxins (18). However, since there are at least 20 Vβ families in human (2, 21), we were limited by the lack of a complete set of anti-Vβ mAbs. We have, therefore, modified the methodology of the polymerase chain reaction (PCR) (22, 23) to allow us to estimate in a population of T-cell blasts the proportion of β-chain mRNA containing any particular Vβ element. By using this method we have established the principal Vβ elements involved in the response of human T cells to five different *S. aureus* toxins. In some cases the response to a particular toxin is caused by T cells bearing homologous Vβ elements in mouse and man. These results have implications for the mode of action of these toxins, and the quantitative PCR reaction we have developed may serve well in the rapid estimation of human T-cell repertoires in a number of diseases.

MATERIALS AND METHODS

Preparation of RNA and cDNA Synthesis. Total RNA was prepared from anti-CD3-stimulated peripheral T cells as described (18, 24). Two micrograms of total RNA was used for the synthesis of first strand cDNA using reverse transcriptase (Amersham) and random hexanucleotides. The reaction was stopped by heating for 5 min at 95°C before PCR.

Amplification of cDNA by PCR and Quantitation of Amplified Products. One-twentieth of each cDNA sample was coamplified using a Vβ-specific primer with a Cβ primer and two Cα primers (Table 1) at a final concentration of 0.3 μM in each reaction. The amplification was performed with 2.5 units of Taq polymerase (*Thermus aquaticus* DNA polymerase) (Perkin–Elmer) and a Cetus/Perkin–Elmer thermocycler under the following condition: 95°C melting, 55°C annealing, and 72°C extension for 1 min each. For quantitation of amplified products, coamplication was performed with 5′ ^{32}P-labeled reverse primers (about 5×10^5 cpm each). The amplified products were separated on 2% agarose gels, dried, and exposed to x-ray film. The autoradiograms were used to identify and cut out the Vβ-Cβ and Cα bands. Each band was assayed for radioactivity by liquid scintillation spectroscopy. In control experiments, the relative amplification efficiency was calculated essentially as described by Chelly *et al.* (23).

RESULTS

Vβ Usage Estimated by PCR. Among the at least 20 different families of human Vβ genes, at least 46 different members of these families have been cloned and sequenced (2, 21, 25). To analyze human T-cell Vβ usage, we synthesized 22 different Vβ-specific oligonucleotides for use as 5′ sense primers for PCR. Their sequences, and the Vβs that they would be expected to amplify, are shown in Table 1. All Vβs indicated as amplified have sequences matching their corresponding primers exactly. There may have been other Vβ genes amplified with these primers. For example, the Vβ6

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Abbreviations: PCR, polymerase chain reaction; MHC, major histocompatibility complex; TCR, T-cell receptor; mAb, monoclonal antibody; SEB, SEC2, and SEE, *S. aureus* enterotoxins B, C2, and E, respectively; ExT, exfoliating toxin; TSST, toxic shock syndrome toxin 1.

Table 1. Sequence of primers used for PCR

Primer	5′ → 3′ sequence	Member*
Vβ1	GCACAACAGTTCCCTGACTTGCAC	1.1-2
Vβ2	TCATCAACCATGCAAGCCTGACCT	2.1-3
Vβ3	GTCTCTAGAGAGAAGAAGGAGCGC	3.1-2
Vβ4	ACATATGAGAGTGGATTTGTCATT	4.1-3
Vβ5.1	ATACTTCAGTGAGACACAGAGAAAC	5.1
Vβ5.2-3	TTCCCTAACTATAGCTCTGAGCTG	5.2-3
Vβ6.1-3	AGGCCTGAGGGATCCGTCTC	6.1-3
Vβ7	CCTGAATGCCCCAACAGCTCTC	7.1-2
Vβ8	ATTTACTTTAACAACAACGTTCCG	8.1-4
Vβ9	CCTAAATCTCCAGACAAAGCTCAC	9.1
Vβ10	CTCCAAAAACTCATCCTGTACCTT	10.1-2
Vβ11	TCAACAGTCTCCAGAATAAGGACG	11.1-2
Vβ12	AAAGGAGAAGTCTCAGAT	12.1-2
Vβ13.1	CAAGGAGAAGTCCCCAAT	13.1†
Vβ13.2	GGTGAGGGTACAACTGCC	13.2†
Vβ14	GTCTCTCGAAAAGAGAAGAGGAAT	14.1†
Vβ15	AGTGTCTCTCGACAGGCACAGGCT	15.1
Vβ16	AAAGAGTCTAAACAGGATGAGTCC	16.1
Vβ17	CAGATAGTAAATGACTTTCAG	17.1
Vβ18	GATGAGTCAGGAATGCCAAAGGAA	18.1
Vβ19	CAATGCCCCAAGAACGCACCCTGC	19.1
Vβ20	AGCTCTGAGGTGCCCCAGAATCTC	20.1
3′Cβ	TTCTGATGGCTCAAACAC	
5′Cα	GAACCCTGACCCTGCCGTGTACC	
3′Cα	ATCATAAATTCGGGTAGGATCC	

The size of amplified products (Vβ bands) by Vβ and 3′Cβ primers ranged from about 170 to 220 base pairs (bp). The size of the amplified cDNA (Cα band) by 5′Cα and 3′Cα primers was about 600 bp. The 3′Cβ primer used in this study matches exactly both Cβ1 and Cβ2 DNA. The sequences of Vβ, Cβ, and Cα are from previously published reports (26–32).

*Members of each Vβ family that have identical sequences as the corresponding primer are listed.

†Vβ13.1, Vβ13.2, and Vβ14.1 have also been called Vβ12.3, Vβ12.4, and Vβ3.3, respectively (2, 30).

primer matches Vβ6.4 except for one nucleotide, and further experiments will be needed to find out if Vβ6.4 is amplified using this primer. Altogether, all of these primers would be expected to cover at least 39 of the 46 sequenced human genes. Each Vβ-specific oligomer was picked to have roughly the same G+C content and to be located at relatively the same position in Vβ.

Total RNA was prepared from human peripheral T cells stimulated by anti-CD3 antibody or one of five different *S. aureus* toxins [SEB, *S. aureus* enterotoxins C2 and E (SEC2 and SEE), exfoliating toxin (ExT), and toxic shock syndrome toxin 1 (TSST)]. At the time of analysis these populations contained 50–90% T-cell blasts as judged by flow cytometric analysis. A single-strand cDNA was prepared for mRNA phenotyping (26, 33) and aliquots of cDNA from each sample were amplified with each of the 22 5′ Vβ-specific sense primers and the 3′ Cβ-specific antisense primer. As an internal control, TCR α-chain mRNA was coamplified in the same tube. Amplification was performed with 25 cycles, a limited number used to ensure that the amount of product synthesized was proportional to the amount of Vβ mRNA in the original preparation. The specificity of each Vβ-specific primer was determined by the size of its amplified product and hybridization to the amplified products of specific probes (not shown). The amplification efficiencies of four of the primer sets (5′Cα to 3′α, or 5′ Vβ2, Vβ3, or Vβ8 to 3′Cβ) were determined as described by Chelly *et al.* (23). The average efficiency ranged about 46–48%. For each sample the number of cpm in the Vβ band was normalized to those found in the Cα band.

We wished to find out whether or not the relative incorporation in this PCR reaction was proportional to the number of cells in the responding population expressing a particular Vβ element. However, we had to consider two possible sources of error. The first of these was contribution from unstimulated T cells. We reasoned that, since mRNA levels are extremely low in unstimulated T cells compared to T-cell blasts, the contribution from unstimulated cells would only become a problem when the proportion of blasts expressing a particular Vβ was very low compared to the unstimulated cells. Second, since all T cells have the potential to rearrange the β locus on both chromosomes, transcription of Vβ mRNA from a nonproductively rearranged chromosome in at least some T cells might confuse the analysis. Since nonfunctional mRNA could be expected to be at a low level due to its instability, we reasoned again that this mRNA may only present a problem in cases where a particular Vβ element was poorly expressed in the blast population.

In order to test these assumptions, we determined the actual percentage of T-cell blasts expressing Vβ5.2/3, Vβ8, and Vβ12 in the various samples using flow cytometry and anti-Vβ mAbs (18) prior to preparing mRNA. When the normalized PCR incorporations for Vβ5.2-3, Vβ8, and Vβ12 for these samples were plotted in a log–log plot against the percentage of T-cell blasts staining with these anti-Vβ mAbs, a linear relationship was obtained (Fig. 1), with the data from three different experiments indistinguishable. This relationship was most evident for values >1%. Below about 1% Vβ expression or a normalized PCR incorporation of about 30, the correlation was lost. We concluded that contributions from unstimulated T cells and nonproductively rearranged β genes were insignificant when Vβ expression in the blasts was >1%. Therefore, we used the data plotted in Fig. 1 as standard curve to analyze expression of Vβs for which antibody was available, estimating the percentage of Vβ expression from the normalized PCR incorporation. As summarized in Table 2, the normalized PCR value represented the actual percentage of T cells very closely (20% standard error), at least in the case of Vβ5.2-3, Vβ8, and Vβ12, of which the abundance of T cells was analyzed by available antibodies. This close relationship was reproducible in three different individuals (see Fig. 3).

Dominant Vβ Usage in the Response to *S. aureus* Toxins. This PCR methodology was used to analyze the expressio

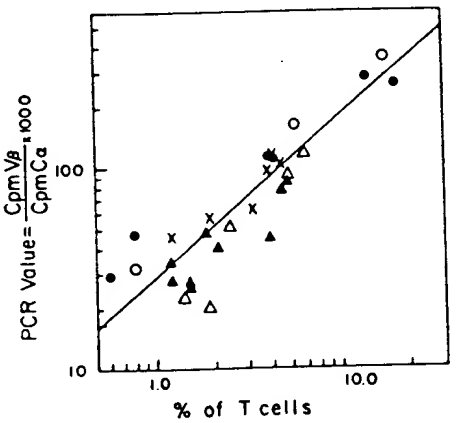

FIG. 1. Standard curve used for the normalization of PCR values to the percentages of T cells bearing particular Vβs in mixed population. T cells were stimulated with anti-CD3 antibody or one of five different *S. aureus* toxins and portions of them were analyzed by flow cytometry as described (18). The PCR value was obtained as indicated on the ordinate—i.e., the cpm in the Vβ band were normalized to those in the Cα band, the internal control. The PCR value and actual percentage of T cells bearing each Vβ were plotted on a log–log graph. X, Vβ5.2-3; ○ and ●, Vβ8; △ and ▲, Vβ12. Open and closed symbols represent the results of two independent experiments.

Table 2. Vβ expression in T cells responding to different stimuli

Vβ	Normal T cells, % Vβ (mAbs)	Anti-CD3 Raw PCR value	Anti-CD3 % Vβ PCR	Anti-CD3 % Vβ mAbs	SEB PCR value	SEB % Vβ PCR	SEB % Vβ mAbs	SEC2 CR value	SEC2 % Vβ PCR	SEC2 % Vβ mAbs	SEE PCR value	SEE % Vβ PCR	SEE % Vβ mAbs	ExT PCR value	ExT % Vβ PCR	ExT % Vβ mAbs	TSST PCR value	TSST % Vβ PCR	TSST % Vβ mAbs
1		88	3.8		20	<1.0		32	1.1		40	1.5		37	1.3		33	1.2	
2		180	9.0		31	1.1		31	1.1		41	1.5		271	14.6		693	45.0	
3		156	7.5		383	22.1		87	3.7		50	1.9		109	4.9		82	3.5	
4		57	2.3		14	<1.0		11	<1.0		16	<1.0		22	<1.0		80	3.4	
5.1		97	4.3		72	3.0		26	<1.0		164	8.0		69	2.8		15	<1.0	
5.2-3	3.2	105	4.7	4.4	61	2.4	3.2	46	1.7	1.2	57	2.3	1.9	98	4.3	3.8	118	5.4	4.0
6.1-3		160	7.8		54	2.1		88	3.8		263	14.1		152	7.3		167	8.2	
7		211	10.8		44	1.7		89	3.8		107	4.8		108	4.9		103	4.6	
8	4.2	168	8.2	5.3	35	1.3	0.2	28	1.0	0.2	349	19.8	15.7	32	1.1	0.8	26	<1.0	0.3
9		55	2.2		10	<1.0		29	1.0		5	<1.0		76	3.2		39	1.4	
10		27	<1.0		11	<1.0		16	<1.0		24	<1.0		15	<1.0		32	1.1	
11		37	1.3		8	<1.0		11	<1.0		11	<1.0		10	<1.0		12	<1.0	
12	1.5	51	2.0	2.4	93	4.1	4.9	120	5.5	5.9	15	<1.0	0.3	20	<1.0	1.9	23	<1.0	1.4
13.1		181	9.0		48	1.8		172	8.5		16	<1.0		31	1.1		16	<1.0	
13.2		67	2.7		55	2.2		115	5.2		33	1.2		30	1.0		25	<1.0	
14		81	3.4		137	6.5		157	7.6		34	1.2		47	1.8		40	1.5	
15		33	1.2		99	4.4		94	4.1		14	<1.0		24	<1.0		26	<1.0	
16		23	<1.0		8	<1.0		13	<1.0		25	<1.0		12	<1.0		12	<1.0	
17		51	2.0		136	6.4		123	5.7		29	1.0		35	1.3		32	1.1	
18		59	2.4		19	<1.0		14	<1.0		129	6.0		68	2.8		70	2.9	
19		76	3.2		41	1.5		22	<1.0		53	2.1		37	1.3		35	1.3	
20		80	3.4		118	5.4		186	9.3		42	1.6		39	1.4		48	1.8	
Total			91.2			66.0			63.1			67.0			55.1			82.4	

Raw PCR values show the normalized cpm obtained as described in the legend to Fig. 1. The PCR data represent the calculated percentage of T cells bearing each Vβ read off the standard curve shown in Fig. 1. PCR values <30 were considered as <1% (see text).

Vβ5.2-3, Vβ8, Vβ12, and 19 other Vβs or Vβ families in normal peripheral T cells stimulated with the various toxins. T cells stimulated with anti-CD3 were used as a control, since our previous experiments (18) showed that stimulation with anti-CD3 did not significantly change the percentages of T cells bearing particular Vβs from that seen in the starting population. Some of our results are shown in Fig. 2. The results of a complete analysis of the response of T cells from a single individual to five different *S. aureus* toxins are summarized in Table 2.

Some Vβ families were used more abundantly than others by normal peripheral T cells. Members of the Vβ2, -3, -6, -7, and -8 families and Vβ13.1 were expressed by >50% of total T cells. Such a finding was perhaps not unexpected for Vβ6 and Vβ8, which are part of large families of Vβs, but is more surprising for Vβ13.1, which appears to be the product of a single gene. The uneven expression of Vβs by human peripheral T cells did not appear to be idiosyncratic for this individual or determined by MHC, since similar frequencies were seen for two other unrelated donors tested (see below, Fig. 3).

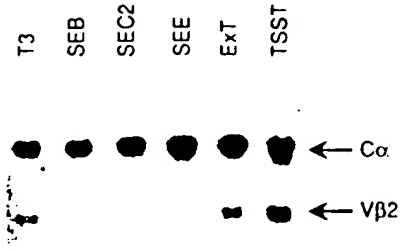

FIG. 2. Autoradiograms of coamplified cDNAs of human TCR transcripts after stimulation with anti-CD3 antibody or five different *S. aureus* toxins. Amplified products were electrophoresed on 2% agarose gels, dried, and exposed to x-ray film for 1 hr at −70°C with an intensifying screen. T3, anti-CD3 antibody.

Complete analysis of the expression of mRNA for all 20 families of human TCR Vβ genes showed clearly that all of the toxins preferentially stimulated T cells expressing particular Vβs; moreover, the pattern of stimulation was different for each toxin. A number of striking new associations were found. Most dramatically, Vβ2-bearing cells were highly enriched by stimulation with TSST. About 50% of the T cells in TSST-stimulated T-cell blasts had Vβ2. As we have previously shown (18), SEB stimulated T cells bearing Vβ12, but this analysis also revealed stimulation of T cells bearing Vβ3, Vβ14, Vβ15, Vβ17, and perhaps Vβ20 by SEB. The related toxin, SEC2, also stimulated T cells expressing Vβ12, Vβ14, Vβ15, Vβ17, and Vβ20 but not those expressing Vβ3. SEE stimulated T cells bearing members of the Vβ8 family, as we have previously shown, but also increased the proportion of Vβ5.1$^+$, Vβ6.1-3$^+$, and Vβ18$^+$ cells.

By using this method, we were able to estimate roughly the percentage of all T cells in a given population that could be accounted for by summing those bearing the different Vβs we measured. As shown in Table 2, this percentage was about 90% for T cells stimulated with anti-CD3, suggesting that our estimate that the Vβ oligonucleotides would prime for expansion of mRNAs encoded by 39 of the 46 human Vβ genes is not too far off, certainly not by an order of magnitude. This suggests that the 46 known Vβ sequences probably cover most of the human genes. The quantitative PCRs accounted for a lower percentage of blasts stimulated by some of the toxins, in particular, ExF. It is possible that this toxin predominantly stimulates T cells bearing Vβs not covered by our primers.

Some of the most dramatic associations in Table 2 were tested in two additional individuals to see how general the phenomena were (Fig. 3). In their responses to these toxins the three individuals behaved almost identically. For exam-

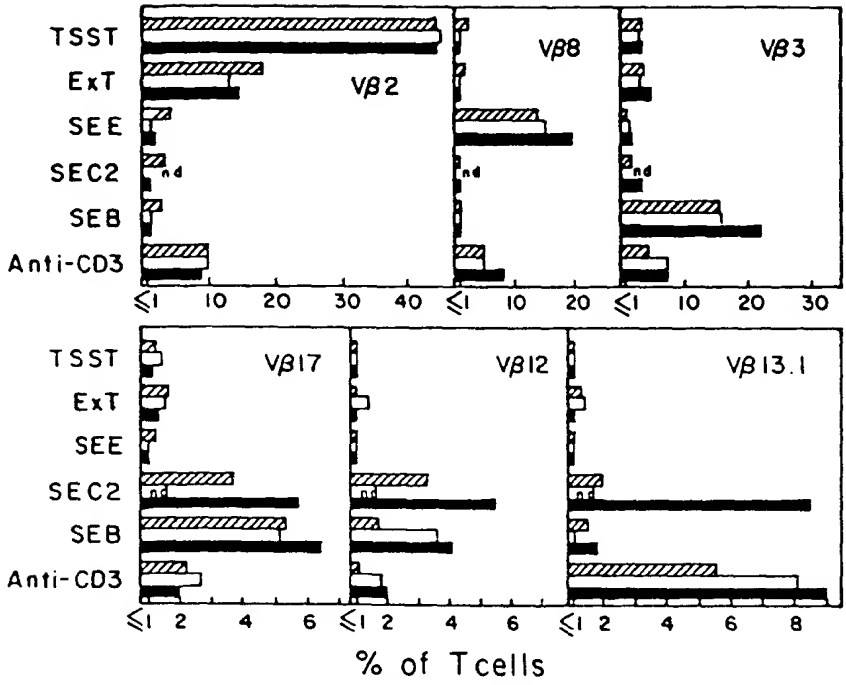

FIG. 3. Vβ-specific stimulation of *S. aureus* toxins in three different individuals. The percentages of T cells bearing each Vβ in different samples were calculated as described in the legend to Fig. 1. nd, Not determined.

ple, Vβ2$^+$ T cells were enriched by TSST to almost the same level of 45% in every case. Similarly, in all three individuals, SEB stimulated T cells bearing Vβ3 and SEE stimulated T cells bearing Vβ8.

Comparison of Vβ Usage in Mice and Humans. The similarities between mice and humans in the T-cell response to these toxins are striking. In both cases T cells bearing particular Vβs dominate the response to each toxin. In both cases the discriminatory powers of the toxins can be particularly dramatic. For example, in humans Vβ5.1$^+$ T cells responded to SEE, whereas cells bearing Vβ5.2/3 did not. Similarly, in the mouse several toxins can distinguish among the members of the Vβ8 family (J.C., J.K., and P.M., unpublished data). This member-specific response to superantigens has also been seen in mice for the endogenous superantigen Mls-1^a, which stimulates T cells bearing Vβ8.1 but not those expressing Vβ8.2 or Vβ8.3 (13).

Extensive sequence analysis of Vβ genes from mouse and man shows that there are some homologues, both by primary sequence and by their relative location in the Vβ gene complex (2, 21, 25). We compared the stimulation patterns by the different toxins of these homologues by using data for mouse Vβ stimulation by toxins (ref. 15; J.C., J.K., and P.M., unpublished data). As indicated in Table 3, in some cases T cells bearing homologous Vβs show a similar pattern of response to the toxins. ExT and especially TSST, for example, stimulated T cells bearing human Vβ2 and mouse T cells bearing the most analogous Vβ, 15. Human T cells expressing members of the Vβ12, -14, -15, and -17 families all showed a tendency to respond to SEB and SEC2 but not ExT or TSST. This property was shared by their closest murine relatives, mouse Vβ8.1, Vβ8.2, and Vβ8.3. However, similar response patterns by T cells bearing homologous Vβs were not always seen. For example, T cells bearing murine Vβ3 responded to most of these toxins; however, those bearing the closest human analog, Vβ10, did not. Even with all this information in hand, a close examination of the primary amino acid sequences of the human and mouse Vβ elements has not yet revealed the essential residues responsible for toxin specificity.

In comparing mouse and man, the most striking difference to emerge thus far in our studies is the apparent lack mechanisms limiting Vβ expression in humans. In the mou despite the potential for expression of >20 Vβ elements in the species as a whole (1–4), various mechanisms limit Vβ expression in individual mice. In some strains large genetic deletions have eliminated about half of the Vβ gene elements (34). Other Vβ gene elements are often inactivated by point

Table 3. Correlations between mouse and human Vβ usage in response to *S. aureus* toxins

Species	Vβ	% homology to mouse	Enrichment* SEB	SEC2	SEE	ExT	TSST
Mouse	8.1		+	±	−	−	−
	8.2		+	+	−	−	−
	8.3		+	−	−	−	−
Human	14	67	+	+	−	−	−
	12	62	+	+	−	−	−
	13	60	−	±	−	−	−
	15	58	+	+	−	−	−
	11	55	−	−	−	−	
	17	52	+	+	−	−	
Mouse	11		−	−	+	−	
Human	8	71	−	−	+	−	−
	6.1-3	60	−	−	+	±	±
	18	55	−	−	+	±	±
Mouse	15		−	−	−	+	+
Human	2	45	−	−	−	±	+
Mouse	3		+	+	−	+	+
Human	19	67	−	−	−	−	−
	10	56	−	−	−	−	

The indicated percent homologies are based on primary amino a sequences.

*Enrichment in response to the indicated toxin. ±, Percentage of T cells was not significantly changed before and after stimulation with the indicated toxin; +, >1.5-fold enrichment of T cells after stimulation with the indicated toxin; −, no enrichment.

mutations (35). Most ingeniously, in many strains of mice, self-superantigens, expressed during T-cell development, lead to the deletion f T cells bearing particular Vβ elements during the establishment of self-tolerance (9–14). We have proposed that these mechanisms, which lead to limited Vβ expression in individual mice, may be a protective evolutionary response to the pressure exerted by bacterial toxins, so that in a population of mice some individuals will be relatively resistant to the effects of any particular toxin superantigen. No evidence for widespread similar mechanisms in humans has emerged thus far from the limited number of individuals examined (18, 36). Thus large genetic deletions have not been found nor have self-superantigens that cause elimination of T cells bearing particular Vβs been observed. A closer examination of individual members of the Vβ families and of larger human populations, especially those with a much more widespread exposure at an early age to these types of toxins, may be required to observe some of these mechanisms at work in humans.

DISCUSSION

S. aureus toxins are responsible for a good percentage of all food poisoning cases and can also be associated with severe shock and other life-threatening pathology. The mechanism of action of the toxins is unknown. Although related, some members of this set of *S. aureus* toxins are very different in primary structure (37–41). Whether their common property of massive T-cell stimulation indicates an essential role for T cells in their mechanism of action has been an open question. Our finding that each toxin stimulates T cells through a different set of Vβ elements suggests strongly that, rather than some common feature of their primary sequence, it is their ability to stimulate a large number of T cells that is the evolutionarily conserved feature of their structure. This would seem to support the notion that T-cell stimulation is critical to their function. Given this, and the perhaps disadvantageous consequences of T-cell stimulation by bacterial products, it is surprising that related human and mouse Vβs have retained the property of responding to particular toxins in spite of large (50%) sequence divergencies. Perhaps this conservation has occurred because the toxin binding loops of Vβ are also required for some other function—for example, binding to MHC. Alternatively, and less likely perhaps, evolution has selected Vβs for their ability to respond to particular toxins.

In this study we have shown that expression of a large number of different human Vβs can be studied using a quantitative PCR. By using this methodology we have here reported by far the most complete analysis of human Vβ expression published to date. In the absence of a complete set of mAbs, this is the most practical approach to such experiments. The method has the major advantage of speed, since analysis of a sample can be completed within a day or two. We anticipate that this methodology will be useful in the study of human T-cell Vβ expression in various diseases and that the method can also be applied to the study of individual gene expression from other TCR multigene families—for example, Vα, Vγ, or Vδ.

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